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Cellular Consequences of Telomere Shortening in
Histologically Normal Breast Tissues

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14. ABSTRACT We recently demonstrated that telomere lengths were shorter in more aggressive breast cancer subtypes, such as luminal B, HER-2 positive and triple-negative tumors, suggesting tumor telomere length may have clinical utility as a prognostic and/or risk marker for breast cancer. Interestingly, the investigator has shown that moderate telomere shortening occurs specifically in luminal epithelial cells, but not in myoepithelial cells, in the majority of histologically normal terminal ductal lobular units analyzed from patients undergoing reduction mammoplasty, but the extent and degree of shortening varies by the individual. These data imply that there is a reservoir of genetically altered, yet histologically normal, cells within normal breast tissues that may represent fertile ground for tumor development. Since telomere shortening has been associated with cellular senescence and dysfunctional telomeres have been linked to the DNA damage response pathway in cancerous tissues, future planned experiments will assess senescence-associated markers and DNA damage response pathway markers in histologically normal human breast tissues that display either normal or short telomeres (<i>i.e.</i> prior to tumor formation). In addition, the proposed investigation has provided grounding in both basic and translational breast cancer research. The interactive, multidisciplinary research environment has provided the investigator opportunities to interact with pathologists and oncologists, thus fostering future success as an independent breast cancer researcher. To date, all tasks, as outlined in the Statement of Work, are on schedule.					
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Table of Contents

Introduction.....	4
Body.....	5
Key Research Accomplishments.....	5
Reportable Outcomes.....	10
Conclusions.....	10
Appendices.....	11
A – Figure showing luminal telomere shortening.....	11
B – Table showing the extent and degree of telomere shortening.....	12
C – Modern Pathology accepted, in press manuscript.....	13
D – Published abstract for the Department of Pathology Young Investigator’s Day....	36
E – Published abstract for the 3rd Annual Safeway Breast Cancer Retreat.....	37

I. INTRODUCTION

Independent investigations have demonstrated the existence of telomeres shortened cells in histologically normal tissues. Thus, we propose to characterize the cellular consequences of telomere shortening in normal cells. Since telomere shortening has been associated with cellular senescence and dysfunctional telomeres have been associated with activation of the DNA damage response pathway in tumor tissues, including premalignant lesions, we propose to assess senescence-associated markers and DNA damage response pathway markers in histologically normal human breast tissues that display either normal or short telomeres (*i.e.* prior to tumor formation). In addition, the proposed investigation has provided the investigator opportunities to interact with pathologists and oncologists to learn normal and abnormal breast morphology, the strengths and limitations of currently used breast cancer biomarkers, current standards of breast cancer treatment and the scientific rationale for ongoing clinical trials. To date, all tasks, as outlined in the Statement of Work, are on schedule.

Hypothesis and Rationale

Recent investigations have demonstrated that telomere length abnormalities are early and frequent events in the malignant transformation of cancer, including breast, and even occur in seemingly histologically normal breast tissues. In all, these data imply that there is a reservoir of genetically altered, yet histologically normal, cells within normal breast tissues that may represent fertile ground for tumor development. We hypothesize that telomere shortening in normal epithelial cells induce cellular senescence; however, if this pathway is abrogated and the telomeres become critically short and, thereby dysfunctional, then the ends of the chromosomes will be recognized by the cell as double strand breaks and the DNA damage response pathway will be activated. Thus, we propose to further characterize the molecular pathways of telomere shortening in the normal breast epithelium. The novel finding that telomeres shorten in a subset of histologically normal TDLUs has tremendous potential to illuminate the mechanisms that support the initiation of breast cancer and may lead to new prevention and treatment strategies. This hypothesis is being evaluated through three specific aims.

The first specific aim is to compare markers of cellular senescence in histologically normal TDLUs with either normal or short telomeres. We will determine the protein expression levels of senescence-associated markers (p16, p21, pRb, Maspin, macroH2A, Suv39h1, H3K9Me and HP1) by immunohistochemistry in normal tissues obtained from reduction mammoplasties and in matched tumor and histologically normal tissues at defined distances (1cm and 5cm) from the visible tumor margin.

The second specific aim is to determine if the DNA damage response pathway is activated in histologically TDLUs with short telomeres. Protein expression levels for markers in the DNA damage response pathway (pATM, pATR, pChk1, pChk2, PML, BRCA1, 53BP1, and γ H2AX) will be assessed by immunohistochemistry in normal tissues obtained from reduction mammoplasties and in matched tumor and histologically normal tissues at defined distances (1cm and 5cm) from the visible tumor margin.

The third specific aim is to assess cellular senescence and activation of DNA damage response pathway in human mammary epithelial cells (HMEC) from tumor and histologically normal

tissues by artificially shortening telomeres. Telomere Repeat Binding Factor 2 (TRF2) will be over-expressed in primary breast cells by a lentiviral vector to induce telomere shortening and cellular senescence and activation of DNA damage response pathway will be assessed.

II. KEY ACCOMPLISHMENTS

IIa. RESEARCH ACCOMPLISHMENTS

During the first year of this training grant, the investigator has:

- Written and obtained approval from the Office of Human Subjects Research Institutional Review Board at Johns Hopkins (November 12, 2009) and from the Human Research Protection Office of the U.S. Army Medical Research and Materiel Command (January 27, 2010) for collection of the clinical samples to be used in this investigation.
- Established collection protocols of clinical specimens for FISH and IHC experiments utilizing formalin-fixed, paraffin-embedded tissues and for primary cell culture experiments utilizing freshly collected tissue. To date, histologically normal breast tissue from 1 cm and 5 cm from the visible tumor margin has been obtained from 27 women undergoing radical mastectomy. Additionally, histologically normal breast tissue from the right and left breast has been obtained from 14 women undergoing bilateral reduction mammoplasty.
- Demonstrated that telomere shortening occurs specifically in luminal epithelial cells, but not in myoepithelial cells, in histologically normal terminal ductal lobular units (Figure 1, Appendix A). Strikingly, telomere shortening occurs in the majority of histologically normal terminal ductal lobular units analyzed from patients undergoing reduction mammoplasty, but the extent and degree of shortening varies by the individual. (Table 1, Appendix B).
- Evaluated telomere length in 103 cases of invasive breast cancer and correlated telomere length with established molecular markers. The study demonstrated that telomere lengths were shorter in more aggressive breast cancer subtypes, such as luminal B, HER-2 positive and triple-negative tumors, suggesting tumor telomere length may have clinical utility as a prognostic and/or risk marker for breast cancer. (Appendix C).

IIb. TRAINING/EDUCATIONAL ACCOMPLISHMENTS

During the first year of this training grant, the investigator has:

- Interacted and collaborated with oncologists, surgeons, pathologists, molecular epidemiologists and other Ph.D. research scientists who specialize in the research and treatment of breast cancer.

- Attended weekly journal clubs, Oncology translational research seminars, breast cancer seminars, Pathology Grand Rounds, specific meetings of the Hopkins Breast SPORE program and “sign-out” sessions with surgical pathologists.
- Given a seminar on May 4, 2010 at the Breast Cancer Program Seminar series sponsored by the Hopkins Breast SPORE program titled “Telomere Length Alterations: Potential Clinical Utility in Breast Cancer”.
- Presented research findings internally at the Department of Pathology Young Investigator’s Day (April 8, 2010) and at the 3rd Annual Safeway Breast Cancer Retreat: Bench to Bedside to Population (June 25, 2010).
- Attended the American Association of Cancer Research (AACR) special conference titled “The Role of Telomeres and Telomerase in Cancer Research (February 2010; Ft. Worth, TX) and the AACR Annual Meeting (April 2010; Washington D.C.).
- Received experimental training in numerous methods including: fluorescence *in situ* hybridization, immunostaining, histopathology, primary cell culture, study design and statistical analysis.

IIc. PERFORMANCE ACCOMPLISHMENTS

Experimental Milestones

Specific Aim 1 (5 tasks)

Task 1 Months 1-6 In Progress

- Establish validated protocols for IHC using the antibodies for senescence-associated markers.
- Positive (senescent) and negative (non-senescent) control cells have been generated from the WI-38 fibroblast cell line and from human mammary epithelial cells obtained from surgical specimens. Cell blocks have been generated to be used for staining purposes. All senescence-associated antibodies have been identified and commercially obtained.

Task 2a Months 6-30 In Progress

- Clinical specimen collection from the Department of Pathology at The Johns Hopkins Hospital under the guidance of Dr. Pedram Argani. Procure clinical specimens from 50 cases (tumor, 1 cm and 5 cm tissues) that will be formalin-fixed and paraffin-embedded. Approximately two cases will be obtained each month.
- To date, clinical specimens from 27 cases (1 cm and 5 cm) have been procured and formalin-fixed and paraffin-embedded.

Task 2b Months 6-30 In Progress

- Clinical specimen collection from the Department of Pathology at The Johns Hopkins Hospital under the guidance of Dr. Pedram Argani. Procure 50 reduction mammoplasty specimens that will be formalin-fixed and paraffin-embedded. Approximately two cases will be obtained each month.

- To date, clinical specimens from 14 reduction mammoplasty specimens (both from the right and left breasts) have been procured and formalin-fixed and paraffin-embedded.

Task 3 Months 6-30 In Progress

- Perform IHC on the prospectively collected tissues for the senescence-associated markers.
- To date, Telomere-FISH in combination with smooth muscle actin staining (to delineate the myoepithelial cell from the luminal cells) has been completed on all collected tissues. Proper IHC conditions for the senescence-associated markers are currently being developed and validated.

Task 4 Months 24-30 Not Initiated

- Validate findings using well characterized TMAs that contain primary breast cancers and paired normal tissue containing histologically normal TDLUs.

Task 5 Months 24-36 Not Initiated

- Analyze correlations between telomere length and the senescence-associated markers in all tissue types.

Specific Aim 2 (5 tasks)

Task 1 Months 1-6 In Progress

- Establish validated protocols for IHC using the antibodies for DNA damage response markers.
- Positive (5Gy and 10Gy irradiated) and negative (mock irradiated) control cells have been generated from the WI-38 fibroblast cell line. Cell blocks have been generated to be used for staining purposes. All DNA damage response antibodies have been identified and commercially obtained.

Task 2a Months 6-30 In Progress

- Clinical specimen collection from the Department of Pathology at The Johns Hopkins Hospital under the guidance of Dr. Pedram Argani. These will be same specimens collected in specific aim 1. Procure clinical specimens from 50 cases (tumor, 1 cm and 5 cm tissues) that will be formalin-fixed and paraffin-embedded. Approximately two cases will be obtained each month.
- To date, clinical specimens from 27 cases (1 cm and 5 cm) have been procured and formalin-fixed and paraffin-embedded.

Task 2b Months 6-30 In Progress

- Clinical specimen collection from the Department of Pathology at The Johns Hopkins Hospital under the guidance of Dr. Pedram Argani. Procure 50 reduction mammoplasty specimens that will be formalin-fixed and paraffin-embedded. Approximately two cases will be obtained each month.
- To date, clinical specimens from 14 reduction mammoplasty specimens (both from the right and left breasts) have been procured and formalin-fixed and paraffin-embedded.

- Task 3 Months 6-30 **In Progress**
- Perform IHC on the prospectively collected tissues for the DNA damage response markers.
 - To date, Telomere-FISH in combination with smooth muscle actin staining (to delineate the myoepithelial cell from the luminal cells) has been completed on all collected tissues. Proper IHC conditions for the DNA damage response markers are currently being developed and validated.

- Task 4 Months 24-30 **Not Initiated**
- Validate findings using well characterized TMAs that contain primary breast cancers and paired normal tissue containing histologically normal TDLUs.

- Task 5 Months 24-36 **Not Initiated**
- Analyze correlations between telomere length and the DNA damage response markers in all tissue types.

Specific Aim 3 (5 tasks)

- Task 1. Months 1-6 **Not Initiated**
- Establish a lentiviral vector with inducible and controllable expression of Telomere Repeat Binding Factor 2 (TRF2)
 - The lentiviral vector will be established in the upcoming year.

- Task 2a Months 6-30 **In Progress**
- Clinical specimen collection from the Department of Pathology at The Johns Hopkins Hospital under the guidance of Dr. Pedram Argani. These will be same specimens collected in specific aim 1. Procure freshly collected viable breast tissue clinical specimens from 50 cases (tumor, 1cm and 5cm tissues). Approximately two cases will be obtained each month.
 - To date, freshly collected, viable breast tissue (1cm and 5 cm) has been successfully collected from 16 radical mastectomy cases.

- Task 2b Months 6-30 **In Progress**
- Clinical specimen collection from the Department of Pathology at The Johns Hopkins Hospital under the guidance of Dr. Pedram Argani. Procure freshly collected viable breast tissues 50 reduction mammoplasty specimens. Approximately two cases will be obtained each month.
 - To date, freshly collected, viable breast tissue (right and left breast) has been successfully collected from 8 reduction mammoplasty cases.

- Task 3. Months 1-24 **In Progress**
- Establish primary cell cultures from collected specimens.
 - Primary cell cultures have been established from the 16 radical mastectomy cases and from the 8 reduction mammoplasty cases.

- Task 4. Months 6-30 **Not Initiated**
- Infect primary cells from obtained tissues with TRF2 lentiviral vector and monitor telomere length, senescence-associated markers and DNA damage response markers by fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC).

- Task 5. Months 24-36 **Not Initiated**
- Analyze correlations between telomere length, senescence-associated markers and DNA damage response markers.

Education and Training Milestones (7 tasks)

- Task 1. Months 1-3 **Not Initiated**
- Attend the Pathology of Cancer graduate course offered through the Department of Pathology at The Johns Hopkins University School of Medicine.
 - The investigator will take this course in the upcoming year.

- Task 2. Months 1-24 **In Progress**
- Learn to recognize morphology and features of different types of breast cancer under the guidance of Dr. Pedram Argani by attending “sign-out” sessions.
 - The investigator has attended “sign-out” sessions with breast surgical pathologists and has learned to recognize the morphology of different types of breast cancer, as well as precursor lesions such as ductal carcinoma in situ (DCIS) and atypical ductal hyperplasia (ADH). Additionally, the investigator has learned to recognize the morphological differences between different benign breast diseases (BBD) such as proliferative and non-proliferative BBD.

- Task 3. Months 1-36 **In Progress**
- Attend Pathology Grand Rounds to gain understanding of the underlying mechanisms of disease and the implications it has for the diagnosis and treatment of different diseases.
 - The investigator attends the weekly Department of Pathology Ground Rounds.

- Task 4. Months 1-36 **In Progress**
- Attend weekly journal clubs, Oncology translational research seminars and specific breast cancer seminars to gain knowledge of the current understanding of breast cancer research and treatment.
 - The investigator attends various departmental journal clubs, Oncology translational research seminars and seminars specifically on breast cancer research.

- Task 5. Months 1-36 **In Progress**
- Attend specific meetings of the Hopkins Breast SPORE program to interact with clinicians and basic science researchers focused on breast cancer.
 - The investigator routinely attends the weekly meetings of the Hopkins Breast SPORE program and presented his research findings titled “Telomere Length Alterations: Potential Clinical Utility in Breast Cancer” as a research seminar on May 4, 2010.

Task 6. Months 12-36 **In Progress**

- Present ongoing work at local and national meetings
- The investigator presented research findings internally at the Department of Pathology Young Investigator's Day (Appendix D) and at the 3rd Annual Safeway Breast Cancer Retreat: Bench to Bedside to Population (Appendix E).

Task 7. Months 12-36 **In Progress**

- Publish research findings in peer-reviewed journals
- The investigator is the primary author on a manuscript that evaluated telomere length in 103 cases of invasive breast cancer and correlated telomere length with established molecular markers. The study was accepted for publication in *Modern Pathology* and is currently *in press* (Appendix C).

III. REPORTABLE OUTCOMES

Publications:

C.M. Heaphy, A.P. Subhawong, A. Gross, Y. Konishi, N. Kouprina, P. Argani, K. Visvanathan, and A.K. Meeker. Shorter Telomeres in Luminal B, HER-2 and Triple-negative Breast Cancer Subtypes. *Modern Pathology*; *in press* (Appendix C).

IV. CONCLUSIONS

To date, all tasks; as outlined in the Statement of Work are on schedule. The appropriate approvals for the collection of the clinical samples to be used in this investigation have been obtained. Tissue collection protocols have been established and procurement of clinical samples has been initiated. The accrual rates for obtaining tissues from radical mastectomies and reduction mammoplasties are on target. The investigator has demonstrated that telomere shortening occurs specifically in luminal epithelial cells, but not in myoepithelial cells, in histologically normal terminal ductal lobular units and occurs in the majority of histologically normal terminal ductal lobular units analyzed from patients undergoing reduction mastectomy. However, the extent and degree of telomere shortening varies by the individual. Additionally, the investigator has shown that telomere lengths are shorter in the more aggressive breast cancer subtypes, such as luminal B, HER-2 positive and triple-negative tumors, suggesting tumor telomere length may have utility as a prognostic and/or risk marker for breast cancer. A manuscript containing these results has been accepted for publication in the *Modern Pathology*, which is currently in press. The investigator is progressing with all of his educational and training goals.

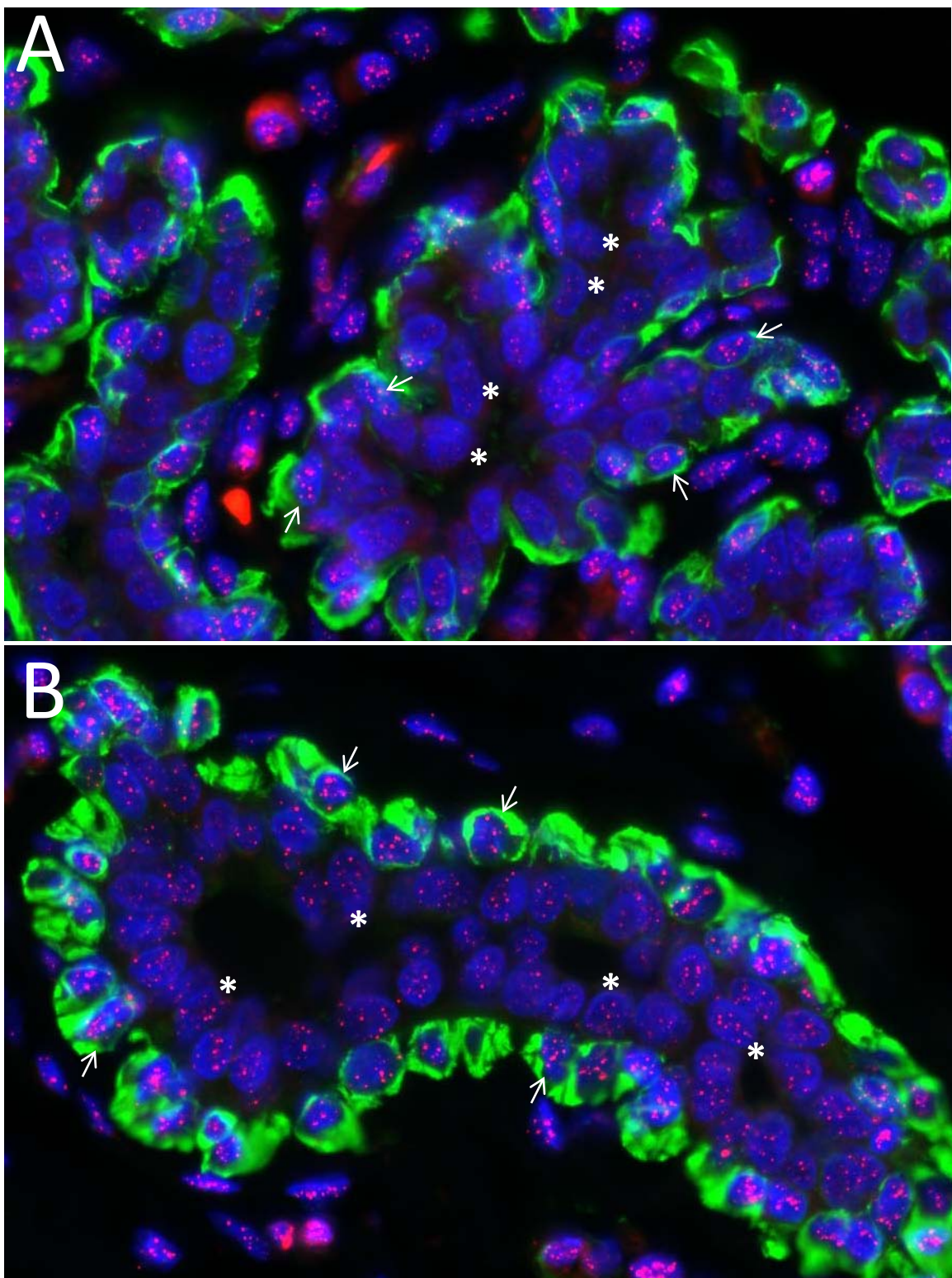


FIGURE 1. Fluorescence *in situ* hybridization (FISH) analysis of telomere length in normal breast tissue obtained from a patient undergoing reduction mammoplasty. (A) A normal breast terminal ductal lobular unit (TDLU) with short telomeres in the luminal cells. (B) A normal breast TDLU with normal length telomeres in all cell types present. The asterisks show luminal cells and the white arrows show myoepithelial cells demarcated by the presences of smooth muscle actin (green). Telomeres (red) and DAPI-stained nuclei (blue) are also shown.

Table 1. Presence of telomere shortening of luminal epithelial cells within histologically normal terminal ductal lobular units (TDLU), large glands and small glands with 14 cases of breast tissue obtained from women undergoing reduction mammoplasty surgeries.

[illegible]

Shorter Telomeres in Luminal B, HER-2 and Triple-negative Breast Cancer Subtypes

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Running Title: Breast cancer subtype telomere lengths

ABSTRACT

Telomeres are nucleoprotein structures that protect chromosome ends from degradation and recombination. Cancers often have critically shortened telomeres, contributing to genomic instability. Many of these tumors are able to activate telomerase to stabilize telomeric ends and achieve a capacity for unlimited replication. Telomere shortening has been reported in *in situ* and invasive carcinomas, including breast, and has been associated with disease recurrence after surgical resection. However, previous studies have not evaluated breast cancer subtypes. The objective of this study was to evaluate telomere lengths in different subtypes of breast cancer. Breast carcinomas (n=103) identified between 2001 and 2010 from patients seen at Johns Hopkins Hospital were categorized into luminal A (n=18), luminal B (n=28), HER-2 positive (n=20) and triple-negative carcinomas (n=37) based on tumor characteristics. Telomere lengths were assessed directly at the single cell level by fluorescence *in situ* hybridization (FISH) and patient groups were compared using Fisher exact tests. ER negative status (p=0.022), PR negative status (p=0.008), HER-2 positive status (p=0.023) and p53 positive status (p=0.022) were associated with shorter telomere length. A larger proportion of luminal A cancers had normal or long telomere lengths as compared to luminal B cases (p=0.002), HER-2 positive cases (p=0.011) or triple-negative cases (p=0.0003). Luminal B, HER-2 positive and triple-negative cases did not differ significantly. Telomere length was shorter in more aggressive subtypes, such as luminal B, HER-2 positive and triple-negative tumors, suggesting tumor telomere length may have utility as a prognostic and/or risk marker for breast cancer.

Keywords: Breast cancer, HER-2, luminal A, luminal B, subtype, telomere, triple-negative

INTRODUCTION

Telomeres, specialized nucleoprotein structures, function to protect and stabilize the ends of eukaryotic chromosomes by preventing chromosome fusions, exonucleolytic degradation, and masking telomere-induced double strand DNA break damage signals. However, telomeres can become critically shortened, and dysfunctional, by several mechanisms including incomplete replication, where telomeres are shortened during each cycle of chromosome replication (1). In normal cells, telomere shortening induces tumor suppressive checkpoint pathways, such as cellular senescence or apoptosis, which halts cell cycle progression before telomeres become destabilized (2, 3). However, abrogation of these checkpoints leads to continued cell division. Consequently, there is a limit to the number of doublings somatic cells can undergo before triggering successive rounds of chromosome breakage-fusion-bridge cycles, thus driving chromosome amplification, and loss or structural rearrangements (4).

It is well established that telomere shortening is present in the majority of *in situ* and invasive carcinomas (5), including breast (6, 7). Thus, telomere shortening is an early event in malignant transformation. In addition to tumor initiation, short dysfunctional telomeres may also affect disease progression. Previous studies have shown reduced telomere length in Grade 3 tumors (8), reduced telomere DNA content, a surrogate for telomere length, correlates with aneuploidy and lymph node metastasis (9), and shorter telomeres were associated with higher stage and histological grade (10). A retrospective study (n=77) demonstrated that short telomeres were associated with tumor size, nodal involvement, TNM stage and was an independent predictor of 5-year overall survival and 5-year breast cancer-free survival. (11). A larger population-based prospective study (n=530) showed that short telomeres conferred a relative hazard of breast

cancer recurrence of 2.88, after adjusting for prognostic factors and adjuvant therapies (12). However, none of these studies took into consideration specific breast cancer subtypes that are now used to help guide treatment decisions.

Four main molecular classes of breast cancer were first identified by gene expression profiling (13). On further validation, these subtypes correlate well with clinical characterization of ER, PR and HER-2 protein expression status (14, 15). These groups are luminal A carcinomas (ER and PR positive, HER-2 negative), luminal B carcinomas (ER or PR positive, HER-2 positive), HER-2 carcinomas (ER and PR negative, HER-2 positive) and triple-negative carcinomas (ER/PR/HER-2 negative). Important for clinical care, these subtypes predict prognosis and therapeutic response (16-18). Luminal A tumors respond well to selective estrogen receptor modulators (SERMs), such as tamoxifen (19). Luminal B tumors tend to be less sensitive to hormonal therapies and thus carry a worse prognosis compared to the luminal A tumors. HER-2 positive cancers tend to be high grade, aggressive, and carry a poor prognosis; however, they respond well to trastuzumab, an anti-HER-2 monoclonal antibody. Conversely, triple-negative carcinomas are extremely aggressive and currently do not respond to any hormonal or antibody-based targeted therapy (20).

The objective of this study was to evaluate telomere lengths in subtypes of breast carcinoma. This was accomplished using a fluorescence *in situ* hybridization (FISH) assay that allows telomere length assessments in formalin-fixed, paraffin-embedded archival material, while providing single cell resolution and keeping intact tissue architecture. We evaluated telomere

length in 103 cases of invasive breast cancer and correlated telomere length with established molecular markers.

MATERIALS AND METHODS

Case Selection

All of the 103 cases evaluated in this study were incident breast carcinomas that were surgically resected at Johns Hopkins Hospital from 2001 to 2010. At the time of resection, specimens were freshly sectioned, fixed overnight in 10% neutral buffered formalin and uniformly processed. Women treated with neo-adjuvant chemotherapy were not included in this study. Clinical characteristics, such as age at diagnosis, weight, ethnicity, parity status, and menopausal status were obtained from the patient's medical records. Pathological characteristics, such as Elston grade and TNM stage, were obtained from the patient's pathology records. This study was approved by the Institutional Review Board of the Johns Hopkins School of Medicine.

Tissue Microarray Construction

For the majority of the cases (n=72), tissue microarrays were constructed as previously described (21, 22). Briefly, each tissue microarray consisted of 99 tissue cores, each 1.4 mm in diameter. These cores were arranged in 9 rows and 11 columns. Column 6 consisted of various unrelated control tissues, leaving 90 cores on the array for breast carcinoma samples. For each carcinoma case, 5 areas were identified on the hematoxylin and eosin slides, punched from the corresponding donor blocks, and placed on the array. Among the 5 samples of each case, we attempted to include normal tissue and carcinoma *in situ* in 1 sample if possible, leaving 4 to 5 cores of invasive ductal carcinoma per case. Any case that displayed a processing artifact was

excluded. Additionally, as not to exhaust the tissue, small size (<1 cm) cases were excluded. For an additional 31 cases, whole sections from surgical specimen blocks were used.

Immunohistochemistry

All immunohistochemistry was performed as previously described (21, 22). The slides were reviewed by two pathologists (A.P.S. and P.A.) to confirm the interpretation of the results. Immunohistochemistry for ER (monoclonal, 1:1 dilution, Ventana) and PR (monoclonal, 1:60 dilution, DAKO) were performed on all cases as part of a routine panel. Cases demonstrating weak, moderate, or strong nuclear labeling for ER or PR in greater than 1% of cells were considered ER positive or PR positive, respectively. Immunohistochemistry for HER-2 was performed on all cases as part of a routine panel using the DAKO Herceptest kit and following the manufacturer's guidelines. Cases were scored using established criteria as 0 or 1+ (negative), 2+ (equivocal), and 3+ (positive). FISH analysis for HER-2 amplification was performed on all cases with 2+ (equivocal) immunohistochemistry results using the Path Vysion kit (Vysis-Abbott Molecular). Cases with either a 3+ (strong positive) immunohistochemistry score or a HER-2 FISH amplification ratio of greater than 4 were considered HER-2 positive. Cases with low-level amplification (ratios 2.2-4.0) were excluded from this study because of their uncertain clinical significance. To determine the basal subtype, CK 5/6 (monoclonal, DAKO) and EGFR (monoclonal, 1:50 dilution, Zymed) immunohistochemistry was performed on whole sections from a subset of cases (29 of 37) which were negative for ER, PR, and HER-2. For CK 5/6, cases were scored on the basis of percentage of positive cells: 1+ (1% to 25%), 2+ (26% to 50%), 3+ (51% to 75%), and 4+ (76% to 100%). Cases with membranous or cytoplasmic labeling in >25% of neoplastic cells were considered positive. For EGFR, cases were scored on the basis of

percentage of positive cells: 1+ (1% to 25%), 2+ (26% to 50%), 3+ (51% to 75%), and 4+ (76% to 100%). Any strong membranous staining for EGFR was considered a positive, generally labeling 10-50% of neoplastic cells. p53 (monoclonal, Ventana) and Ki-67 (monoclonal, Ventana) immunohistochemistry were also performed and only nuclear labeling was scored. For p53, labeling of >30% of nuclei was considered aberrant overexpression, and labeling of less than 30% of nuclei was considered negative for aberrant overexpression; 30% labeling of nuclei was considered equivocal. For Ki-67, labeling of $\geq 20\%$ of nuclei was considered high and labeling of <20% of nuclei was considered low.

Telomere FISH

Telomere lengths were assessed by fluorescence staining for telomeric DNA as previously described (6, 23). Briefly, deparaffinized slides were hydrated through a graded ethanol series, placed in deionized water, followed by deionized water plus 0.1% Tween-20. Slides were then placed in citrate buffer (catalog No. H-3300; Vector Laboratories), and steamed for 14 minutes (Black and Decker Handy Steamer Plus; Black and Decker), removed, and allowed to cool at room temperature for 5 minutes. Slides were then placed in PBS with Tween (catalog No. P-3563; Sigma) for 5 minutes. Slides were thoroughly rinsed with deionized water, followed by 95% ethanol for 5 minutes, and then air-dried. Twenty-five μl of a Cy3-labeled telomere-specific peptide nucleic acid (0.3- $\mu\text{g}/\text{ml}$ peptide nucleic acid in 70% formamide, 10 mmol/L Tris, pH 7.5, 0.5% B/M Blocking reagent (catalog No. 1814-320; Boehringer-Mannheim) was applied to the sample, which was then coverslipped, and denaturation was performed by incubation for 4 minutes at 83°C. Slides were then moved to a dark, closed container for hybridization at room temperature for 2 hours. Coverslips were then carefully removed and the

slides were washed twice in peptide nucleic acid wash solution (70% formamide, 10 mmol/L Tris, pH 7.5, 0.1% albumin (from 30% albumin solution, catalog No. A-7284; Sigma)) for 15 minutes each, then rinsed in PBS with Tween, and thoroughly rinsed in deionized water. Slides were drained and counterstained with 4'-6-diamidino-2-phenylindole (DAPI) (500 ng/ml in deionized water, Sigma Chemical Co. Cat #D-8417) for 5 minutes at room temperature, mounted with Prolong anti-fade mounting medium (catalog No. P-7481; Molecular Probes), and imaged. The peptide nucleic acid probe complementary to the mammalian telomere repeat sequence was obtained from Applied Biosystems, and has the sequence (N-terminus to C-terminus) CCCTAACCCTAACCCTAA with an N-terminal covalently linked Cy3 fluorescent dye. As a positive control for hybridization efficiency, a FITC-labeled peptide nucleic acid probe having the sequence ATTCGTTGGAAACGGGA with specificity for human centromeric DNA repeats (CENP-B binding sequence) was also included in the hybridization solution (24).

Microscopy and Telomere Length Assessment

Slides were imaged with a Nikon 50i epifluorescence microscope equipped with X-Cite series 120 illuminator (EXFO Photonics Solutions Inc., Ontario, CA). Fluorescence excitation/emission filters were as follows: Cy3 excitation, 546 nm/10 nm BP; emission, 578 nm LP (Carl Zeiss Inc.); DAPI excitation, 330 nm; emission, 400 nm via an XF02 fluorescence set (Omega Optical, Brattleboro, VT); Alexa Fluor 488 excitation, 475 nm; emission, 535 nm via a combination of 475RDF40 and 535RDF45 filters (Omega Optical). Grayscale images of representative regions were captured for presentation using Nikon NIS-Elements software and an attached Photometrics CoolsnapEZ digital camera, pseudo-colored and merged. Integration times typically ranged from 500 ms to 800 ms for Cy3 (telomere) and FITC (centromere) signal capture, 50 ms to 100 ms for

the DAPI counterstain. Telomere lengths were qualitatively scored by direct visual assessment of the stained slides, comparing telomere signals from tumor cells to telomere signals from benign cells (stromal cells and/or myoepithelial cells) from the same case. In all cases, signals from benign cells were considered 3+. Telomeres in the tumor cells of different cases ranged from short (0+, 1+, or 2+) to normal (3+) to long (4+, 5+).

Statistical Analysis

For all analyses, the normal and long telomere groups were combined and compared to the short telomere group. Results were compared using two-sided Fisher exact tests. P-values < 0.05 were considered to be significant. SAS 9.2 and JMP[®] statistical packages (SAS Institute, Cary, NC) were used for all analyses.

RESULTS

Clinical and Pathological Characteristics: Table 1 lists the clinical and pathological characteristics of the study population. Of the 103 subjects included in this study, the mean age at diagnosis for all patients was 56 years (range; 30-94 years). The patients were predominantly Caucasian (57%) or African American (31%). Elston grading of the tumors showed that 4% were Grade I, 23% were Grade II and 73% were Grade III. According to the established criteria of the American Joint Committee on Cancer (AJCC 2007), 21% were Stage I, 59% were Stage II, 18% were Stage III and 1% were Stage IV. Interestingly, the short telomere length group tended to be younger and contained a greater proportion of African American women when compared to the normal/long group; however, these differences did not reach statistical significance.

Telomere-FISH in Breast Tumors: Telomere length was qualitatively scored and grouped into short (n=88), normal (n=13) or long (n=2) categories. Figure 1A shows a representative example of a breast cancer with short telomeres as indicated by the diminished telomere signals in the cancer cells when compared to either stromal cells or myoepithelial cells in an adjacent terminal ductal lobular unit. As shown in Figure 1B, the cancer cells from this breast cancer show comparable telomere intensities to that observed in the surrounding benign stroma. Figure 1C, shows an example of a breast tumor with increased telomere signals in the cancer cells when compared to the surrounding benign stromal cells. Table 1 shows the clinical and pathological characteristics for the subset of patients with short telomeres (n=88) and the subset of patients with either normal or long telomeres (n=15).

Hormone Receptor Expression Characteristics: Table 2 shows tumor characteristics including ER status, PR status, HER-2, p53 and Ki-67 stratified by telomere length. ER negative (p=0.022) PR negative (p=0.008), and HER-2 positive (p=0.023) tumors were significantly more likely to have a greater fraction of short telomeres when compared to the normal/long telomere group. In addition, there was a significant increase in the proportion of p53 positive tumors in the short telomere category (p=0.022). Shorter telomere length was also more prevalent in tumors with an increased Ki67 index ($\geq 20\%$ of cells positive), but it did not reach statistical significance.

Subtype Characterization and Telomere Length Assessment: Cases were categorized into one of 4 groups; luminal A, luminal B, HER-2 positive, or triple-negative. Eighteen cases were ER/PR positive/HER-2 negative, and were considered luminal A cases. There were 28 luminal B cases that were HER-2 positive and ER and/or PR positive. Twenty tumors were HER-2 positive

and negative for ER and PR, and were considered HER-2 positive cases. Additionally, there were 37 triple-negative carcinoma cases which were negative for ER, PR, and HER-2. For 29 of the triple-negative cases, immunohistochemical data on CK 5/6 and EGFR expression was available, allowing assessment of the basal phenotype. Eighteen of these cases showed staining for CK 5/6 and/or EGFR (basal) and 11 cases were CK 5/6 and EGFR negative (non-basal). Within this triple-negative group, there was no difference in the proportion of cases with short telomeres between the subset of cases determined to be basal (94%) and non-basal (91%) phenotypes. As shown in Figure 2, the luminal B cancers (93%), HER-2 cancers (90%) and the triple-negative cancers (95%) had an increased proportion of cases with short telomeres when compared to the luminal A cancers (50%); these were all statistically significant differences ($p=0.002$, $p=0.011$ and $p=0.0003$, respectively).

DISCUSSION

The first principal conclusion emerging from this study is the observation that telomere shortening is associated with other established breast cancer prognostic factors. It is well established that breast cancer patients with tumors that are ER and/or PR negative have an increased risk of mortality (25-27). We observed an increased proportion of tumors in the short telomere group that were negative for ER and PR. Although temporality cannot be determined, these results suggest that telomere shortening may contribute to the selection of cells capable of growing in the absence of hormone receptors. Another molecular marker associated with poor breast cancer prognosis is the presence of mutations in the p53 gene, predominantly mis-sense mutations leading to conformational alterations of the protein and accumulation in tumor cell nuclei (28-30). We observed an increased proportion of tumors in the short telomere group that

were p53 positive, suggesting that less aggressive tumors are characterized by normal length telomeres and no mutations in the p53 gene. Conversely, short, dysfunctional telomeres may provide a strong selective pressure for abrogation of the p53 pathway. Normally, critical telomere shortening leads to p53-dependent tumor suppressive cellular responses, such as cellular senescence and apoptosis (2). However, if this checkpoint is abrogated and the telomeres are partially stabilized by the up-regulation of telomerase, then further proliferation occurs and genomic instability may accumulate (31, 32).

The second principal conclusion emerging from this study is that telomere shortening occurs in the vast majority of luminal B, HER-2 and triple-negative tumors, but in a smaller fraction of the luminal A type tumors. A recent large patient cohort demonstrated that luminal A tumors are associated with a lower risk of local or regional recurrence when compared to the other molecular subtypes (18). It has long been recognized that breast adenocarcinomas are characterized by genomic instability. The molecular mechanisms leading to genomic instability are not fully elucidated; however, one potential initiator of genomic instability is telomere dysfunction due to critical telomere shortening (4, 33). Thus, telomere shortening may be reflective of the degree of underlying genomic instability, a feature shared by higher grade HER-2 and triple-negative carcinomas (34). Another possibility is that telomere shortening follows tumor progression as evidenced by the association with higher stage and histological grade (8, 10, 11). It is noteworthy that recent data have demonstrated that not only can short telomeres lead to telomere dysfunction, but abnormally long telomeres may also do so (35, 36). In this context, we previously demonstrated the presence of alternative lengthening of telomeres, a recombination-based mechanism that lengthens telomeres, in three cases of breast carcinoma

(21). Interestingly, all three cases were also HER-2 positive, suggesting a possible common underlying mechanism. The significance of long telomeres in cancers lacking evidence of the alternative lengthening of telomeres phenotype is currently not clear and would need to be addressed in future studies.

This study is the first assessment of telomere lengths in breast cancer subtypes. Additionally, we analyzed telomere lengths directly, at the single cell level, within breast cancer tissues using a FISH assay. Previous studies have measured telomeres using bulk assays such as Southern blot and terminal restriction fragment analysis (8), quantitative PCR (10) or with a chemiluminescent-based slot blot assay that measures telomere DNA content, a proxy of telomere length (9, 11, 12). These types of measurements do not allow for single cell resolution. Study limitations include the absence of small tumors (<1cm), thus selecting for higher stage and histological grade cases, and the limited demographic and lifestyle information, as this was obtained retrospectively. Additionally, the lack of complete data on follow-up time, due to the fact that part, but not all, of the patients' treatment occurred at our Institution, prevented us from determining an association between telomere length and outcome.

In conclusion, we have demonstrated that telomere lengths vary among different subtypes of breast cancer in a manner consistent with their aggressiveness. Prospective studies are needed to further evaluate the usefulness of telomere length as a prognostic and/or predictive marker of disease progression and treatment response within the various subtypes and determine temporality.

DISCLOSURE/CONFLICT OF INTEREST

All authors have no conflicts of interest to declare.

ACKNOWLEDGEMENTS

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Table 1. Clinical and pathological characteristics for all patients and stratified by telomere length			
Characteristic	All Subjects n=103	Telomere Length*	
		Short n=88	Normal/Long n=15
Age at Diagnosis (y)			
Mean (range)	56 (30-94)	55 (30-92)	61 (34-94)
Weight (kg)**			
Mean (range)	79.1 (44.0-163.5)	79.9 (46.8-163.5)	73.8 (44.0-97.7)
Ethnicity			
Caucasian	59	46	13
African American	32	30	2
Other	12	12	0
Parity Status			
Yes	82	73	9
No	20	14	6
Missing	1	1	0
Menopausal Status			
Pre-menopausal	41	35	6
Post-menopausal	60	52	8
Missing/uncertain	2	1	1
Elston Grade			
1	4	1	3
2	24	20	4
3	75	67	8
TNM Stage			
I	22	22	0
II	61	52	9
III	19	13	6
IV	1	1	0

*There are no statistically significant differences in any of the clinical or pathological characteristics between the telomere length groups

**Eighteen women are missing weight data

Table 2. Association between ER, PR, HER-2, p53 and Ki-67 expression characteristics stratified by telomere length			
Characteristic	Telomere Length		p-value
	Short	Normal/Long	
ER Status			
Positive	34	11	0.022
Negative	54	4	
PR Status			
Positive	30	11	0.008
Negative	58	4	
HER-2			
Positive	53	4	0.023
Negative	35	11	
p53*			
Positive	40	2	0.022
Negative	46	13	
Ki-67**			
High ($\geq 20\%$)	70	8	0.082
Low ($< 20\%$)	17	6	

*Two patients with equivocal p53 staining were not included in the analysis

** Two patients are missing Ki-67 data

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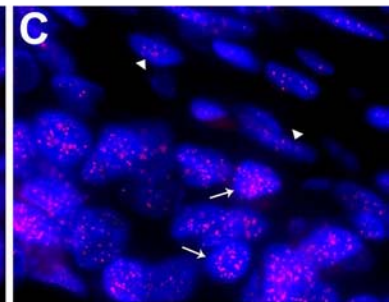
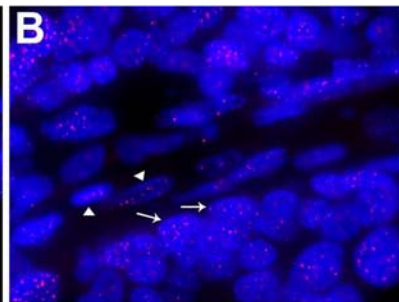
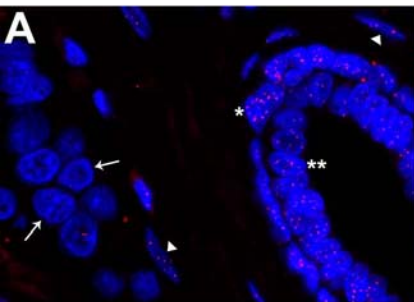
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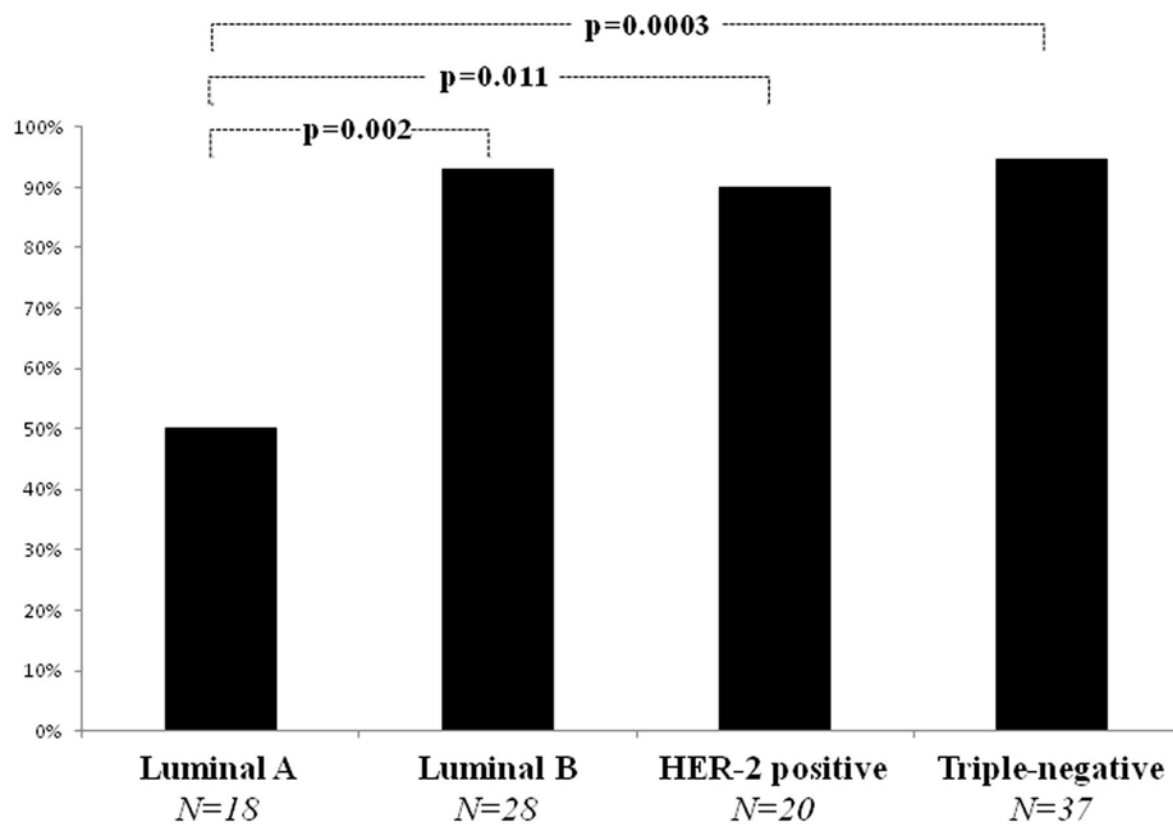
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TITLES AND LEGENDS TO FIGURES

Figure 1. Telomere length analysis by FISH in breast adenocarcinomas. Three representative examples of cases showing short, normal or long telomere lengths in the cancer cells are shown. **(A)** This case shows strikingly diminished telomere signals in the tumor cells as compared to the surrounding benign stroma and in an adjacent terminal ductal lobular unit with myoepithelial (*) and luminal (**) cells. **(B)** This case displays comparable telomere intensities in the tumor cells to those observed in the surrounding benign stroma. **(C)** In this case, the cancer cells show extremely bright telomere signals in the cancer cells when compared to the surrounding benign stroma. In all the images, the DNA is stained with DAPI (blue) and telomere DNA is stained with the Cy3-labeled telomere-specific peptide nucleic acid probe (red). Of note, the centromere DNA, stained with the FITC-labeled centromere-specific peptide nucleic acid probe, has been omitted from the image to emphasize the differences in the telomere lengths. In all panels, the arrows point to cancer cells and the arrowheads point to benign stromal cells. Original magnification $\times 400$.

Figure 2. Proportion of cases with short telomeres among different subtypes of breast cancer. P-values were determined using two-sided Fisher's Exact Tests.





Shorter Telomeres in HER-2 and Triple-negative Breast Cancer Subtypes

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Telomeres are nucleoprotein structures that protect the ends of chromosomes from degradation and recombination. In normal somatic cells, telomeres shorten with each cell cycle until either senescence or apoptosis occurs. Cancers often have critically shortened telomeres, contributing to genomic instability; and many of these tumors are able to activate telomerase to stabilize telomeric ends and achieve a capacity for unlimited replication. Telomere shortening has been reported in *in situ* and invasive carcinomas, including breast, and has been associated with disease recurrence after surgical resection. However, previous studies have not evaluated breast cancer subtypes. The objective of this study was to evaluate telomere lengths in different subtypes of breast cancer. Tissue microarrays consisting of 61 breast carcinomas identified between 2001 and 2007 from patients seen at Johns Hopkins Hospital were categorized into luminal A (n=15), HER-2 positive (n=17) and triple-negative carcinomas (n=29) based on tumor characteristics. Telomere lengths were assessed directly at the single cell level by fluorescence *in situ* hybridization (FISH) and patient groups were compared using Fisher exact tests. ER negative status (p=0.014), PR negative status (p=0.014) and p53 positive status were associated with shorter telomere length. A larger proportion of luminal A cancers had normal or long telomere lengths as compared to HER-2 positive cases (p=0.005) or triple-negative cases (p=0.001). HER-2 positive and triple-negative cases did not differ significantly. Telomere length was shorter in more aggressive subtypes, such as HER-2 positive and triple-negative tumors, suggesting it may have utility as a prognostic and/or risk marker for breast cancer.

Shorter Telomeres in Luminal B, HER-2 and Triple-negative Breast Cancer Subtypes

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Background: Telomeres are nucleoprotein structures that protect chromosome ends from degradation and recombination. Cancers often have critically shortened telomeres, contributing to genomic instability. Many of these tumors are able to activate telomerase to stabilize telomeric ends and achieve a capacity for unlimited replication. Telomere shortening has been reported in *in situ* and invasive carcinomas, including breast, and has been associated with disease recurrence after surgical resection. However, previous studies have not evaluated breast cancer subtypes. The objective of this study was to evaluate telomere lengths in different subtypes of breast cancer.

Methods: Breast carcinomas (n=103) identified between 2001 and 2010 from patients seen at Johns Hopkins Hospital were categorized into luminal A (n=18), luminal B (n=28), HER-2 positive (n=20) and triple-negative carcinomas (n=37) based on tumor characteristics. Telomere lengths were assessed directly at the single cell level by fluorescence *in situ* hybridization (FISH) and patient groups were compared using Fisher exact tests.

Results: ER negative status (p=0.022), PR negative status (p=0.008), HER-2 positive status (p=0.023) and p53 positive status (p=0.022) were associated with shorter telomere length. A larger proportion of luminal A cancers had normal or long telomere lengths as compared to luminal B cases (p=0.002), HER-2 positive cases (p=0.011) or triple-negative cases (p=0.0003). Luminal B, HER-2 positive and triple-negative cases did not differ significantly.

Conclusions: Telomere length was shorter in more aggressive subtypes, such as luminal B, HER-2 positive and triple-negative tumors, suggesting tumor telomere length may have utility as a prognostic and/or risk marker for breast cancer.